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Transport of glucose and fructose in rat hepatocytes at 37°C

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The kinetic parameters for transport of the nonmetabolizable glucose analogue 3-*O*-methyl-D-glucose and the relationship between transport and metabolism of D-glucose and D-fructose were determined in isolated rat hepatocytes at 37°C and pH 7.4. 3-*O*-Methylglucose at a very low concentration (0.1 mM) equilibrated with the intracellular water with a rate constant of 0.41 s^{-1}. K_m for equilibrium exchange entry was 5.5 mM and V_{max} was 2.2 mM · s^{-1} and similar results were obtained when using the zero-trans entry protocol. The rate constant for entry of tracer D-glucose was 0.15 s^{-1} and K_m for glucose was about 20 mM. The phosphorylation rate for D-glucose was much slower than the transport rate. The rate constant for D-fructose entry was about 0.04 s^{-1}, the apparent K_m was about 100 mM and V_{max} about 5 mM · s^{-1}. The concentration dependence of 3-*O*-methylglucose inhibition of labelled fructose transport revealed biphasic kinetics indicating that fructose was transferred by both the glucose transporter and a fructose transporter. At concentrations lower than 1 mM, fructose metabolism appeared to be limited by the transport step.

Introduction

Stereospecific and saturable hexose transport in hepatic cells was first demonstrated by Williams et al. [1] who reported a K_m of 17 mM for uptake of D-glucose in the perfused rat liver at 37°C. On the other hand, Goresky and Nadeau [2] performed similar experiments in dogs and reported a K_m for D-glucose uptake of 120 mM. The calculation of kinetic constants from perfusion experiments is complicated and depends on certain assumptions, and this may be the reason for the variable results.

The properties of the hexose transport system may be assessed more directly by measuring initial uptake rates of the nonmetabolizeable sugar analogue 3-*O*-methyl-D-glucose in isolated rat hepatocytes. This approach was followed by Baur and Heldt [3]. However, these authors found that 1 mM 3-*O*-methyl-D-glucose was equilibrated with the intracellular water by 10 s at 37°C. This was the shortest time employed and the authors were therefore unable to measure initial transport velocities at physiological temperature. Craik and Elliot [4] measured initial 3-*O*-methylglucose transport velocities at 20°C and found a K_m of about 20 mM. Similarly, the inhibition constant of 3-*O*-methylglucose on galactose or fructose uptakes was reported as about 30 mM [5].

The purpose of the present study was to assess the transport kinetics of 3-*O*-methylglucose and some metabolizeable sugars, particularly glucose and fructose at 37°C, to evaluate the rate-limiting step for phosphorylation and metabolism of glucose and fructose, and finally to analyse whether fructose is transferred only via the glucose transporter or whether some might be taken up via a specific fructose transporter.

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Materials and Methods

3-*O*-[^{14}C]Methyl-D-glucose, D-[U-^{14}C]glucose, D-[2-^{3}H]glucose, 2-deoxy-D-[1-^{14}C]glucose, D-[U-^{14}C]fructose, D-[1-^{14}C]galactose, D-[1-^{14}C]mannose and $^{3}H_2O$ were purchased from Amersham International, and L-[1-^{3}H]glucose from New England Nuclear. 3-*O*-Methylglucose, D-glucose, 2-deoxy-D-glucose, L-glucose, D-mannose and D-galactose were from Sigma and D-fructose was from Merck. Percoll was from Pharmacia and collagenase (CLS, 218 U/mg) was from Cooper Biomedical. Bovine serum albumin (fraction V) and dexamethasone were from Sigma. Phloretin from K & K laboratories was dissolved in ethanol/dimethylsulphoxide (7 : 3 v/v) to make a stock solution of 82.5 mg/ml (0.3 M). Salts (analytical grade) were from Merck.

Male Wistar rats weighing between 190 and 250 g were fasted for 24 h except when noted. Hepatocytes were isolated by a modification [6] of the method of Berry and Friend [7]. Briefly, the animal was anesthetized with ether, the liver was flushed in situ through the portal vein with Ca^{2+}-free Hanks-bicarbonate buffer. It was then excised and perfused in vitro with the Ca^{2+}-free buffer for 5 min. Collagenase (0.5 g/l) and $CaCl_2$ (2.5 mM) was then added to the perfusate. After about 30 min, the liver was stripped free of the capsule and the cells were released into a buffer containing (in mM): Na^+ 127, K^+ 4.7, Ca^{2+} 2.5, Mg^{2+} 1.25, Cl^- 134, $H_2PO_4^-/H_2PO_4^{2-}$ 2.5, SO_4^{2-} 1.25 and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) 25. The cells were layered on top of a preformed linear Percoll gradient adjusted to 1.005–1.110 kg/l in Hepes buffer. After centrifugation for 5–10 min at approx. $4000 \times g$, the viable hepatocytes were isolated from a band at 1.070 kg/l [8] and washed twice with Hepes buffer. More than 96% of the cells excluded Trypan blue. Cells from fasted rats did not release glucose into the medium.

Transport assays were carried out at 37°C and pH 7.4, essentially as described for cultured lymphocytes [9] and polymorphonuclear leukocytes [10]. In brief, 45 μl of the hepatocyte suspension (7% v/v) was squirted on to 15 μl buffer containing isotopically-labelled sugar and unlabelled sugar when needed. Timing was assisted using a metronome. Incubations were terminated by the addition of 3.5 ml stopping solution containing 0.3 mM phloretin. The cells were pelleted by centrifugation at about $4000 \times g$ for 1 min, the supernatant was discarded and the procedure was repeated once. Finally, 2.5 ml of scintillation fluid was added and radioactivity determined. Blank values, determined by the addition of stopping solution before the cells, were subtracted from all measurements and they contained about 8% of the counts present in the cell pellet when the cells were allowed to equilibrate with labelled 3-*O*-methylglucose for 5 min.

In some experiments, distribution spaces for $^{3}H_2O$, ^{14}C-labelled 3-*O*-methylglucose and ^{3}H-labelled L-glucose were determined by transferring 100 μl suspension of incubated cells (without wash) to 500 μl microfuge tubes containing 100 μl dibutylphthalate/dinonylphthalate (3 : 1, v/v) (*d* 1.0245) followed by centrifugation. The tubes were cut and radioactivity in the pellet and supernatant fractions was measured.

The rate of glucose metabolism was determined by incubating hepatocytes with [2-^{3}H]glucose at 37°C followed by centrifugation in microfuge tubes. The supernatant was applied to a 0.7×4 cm anion-exchange column (Bio-Rad AG1-X8) as described [10]. The $^{3}H_2O$ and [2-^{3}H]glucose were eluted by washing with 2.5 ml 0.1 mM D-glucose in water. The eluate was applied to a borate column which retains the glucose [11] and $^{3}H_2O$ was eluted by washing with 2.5 ml water.

All experiments were performed at least four times with essentially the same results.

Results

3-O-Methylglucose transport

The intracellular distribution space for ^{14}C-labelled 3-*O*-methylglucose was 20.2 ± 3.0 μl per 10^7 hepatocytes after incubation for 2 min as compared with a $^{3}H_2O$ space of 21.1 ± 2.7 μl (1 S.D., $n = 5$). The cell-associated ^{14}C-activity was not increased when cells were incubated for 30 min indicating that 3-*O*-methylglucose is not converted to metabolic products trapped inside the cell (e.g. phosphorylated). Fig. 1 shows that the equilibration of 0.1 mM 3-*O*-methylglucose with its intracellular distribution space followed an ex-

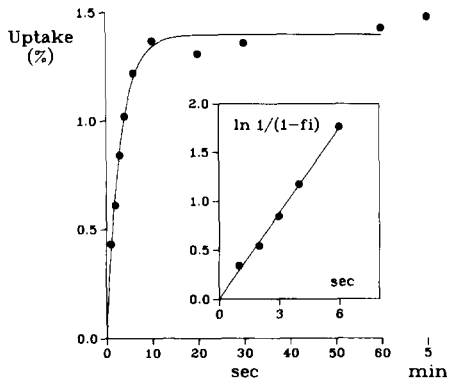

Fig. 1. Time-course of 0.1 mM 3-*O*-methylglucose equilibrium exchange entry. Hepatocytes were preincubated for 10 min with 0.1 mM 3-*O*-methylglucose at 37°C. 45 μl cell suspension was squirted on to 15 μl buffer containing 0.1 mM 3-*O*-methylglucose plus ^{14}C-labelled 3-*O*-methylglucose. The ordinate shows the per cent uptake of 3-*O*-methylglucose and the data are corrected for tracer in the trapped extracellular volume. The inset shows a logarithmic transformation of the fractional filling (f_i), i.e. the fraction of the intracellular distribution space filled at a given time. The rate constant (v/S) was calculated by linear regression as 0.33 s^{-1}. Each point is the mean of four replicates.

ponential curve as previously shown at 20°C [4]. The mean rate constant of entry was 0.41 ± 0.05 s^{-1} (1 S.D., $n = 13$). In contrast, the rate constant of L-glucose entry was about $0.50 \cdot 10^{-3}$ s^{-1} (cf. Table I). Thus, 3-*O*-methylglucose at a very low concentration equilibrated with almost the entire waterspace with a half time of about 1.7 s.

Fig. 2 shows the concentration dependence of 3-*O*-methylglucose equilibrium exchange entry, i.e. inside and outside 3-*O*-methylglucose concentrations are equal. K_m was 5.5 and V_{max} was 2.2 $mM \cdot s^{-1}$. Fig. 2 also shows data for zero-trans entry (i.e. the inside 3-*O*-methylglucose concentration is initially zero) yielding $K_m = 5.1$ mM and $V_{max} = 2.0$ $mM \cdot s^{-1}$. The inset of Fig. 2 compares directly the transport of 10 mM 3-*O*-methylglucose according to the equilibrium exchange and zero-trans entry modes and it appears that the initial uptake rates are closely similar.

Thus, the kinetic constants for zero-trans and equilibrium exchange entry are indistinguishable in agreement with previous results at 20°C [4].

TABLE I

INHIBITION OF 3-*O*-METHYLGLUCOSE TRANSPORT BY ALDOHEXOSES

45 μl hepatocyte suspension was squirted on to 15 μl buffer containing ^{14}C-labelled 3-*O*-methylglucose and unlabelled sugar. Incubations were timed to give approx. 25% accumulation of the tracer in the intracellular distribution space. The K_i values were obtained from the intercept with the abscissa, cf. Fig. 2. The rate constants of entry (v/S) were calculated from similar incubations using the ^{14}C-labelled aldohexoses, cf. Fig. 1. They equaled V_{max}/K_m, since the concentrations of labelled sugars were 20 μM or less. Each value is the mean of three experiments ± 1 S.D.

Sugar analogue	K_i (mM)	v/S (s^{-1})
D-Glucose	18.6 ± 3.2	0.15 ± 0.03
2-deoxy-D-glucose	20.1 ± 5.6	0.22 ± 0.01
D-galactose	53.5 ± 5.7	0.05 ± 0.01
D-Mannose	57.6 ± 5.7	0.05 ± 0.01
L-Glucose	> 100	$< 5 \cdot 10^{-4}$

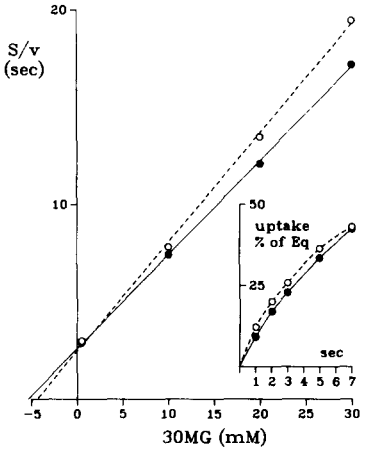

Fig. 2. Concentration dependence of 3-*O*-methylglucose (30MG) entry. The incubations were timed to give approx. 25% accumulation of ^{14}C-labelled 3-*O*-methylglucose in the intracellular distribution space. The experiments were carried out either according to the equilibrium exchange protocol (●———●), cf. legend to Fig. 1, or according to the zero-trans protocol (○———○) where both labelled and unlabelled 3-*O*-methylglucose are present only in the extracellular buffer at time zero. The data are plotted according to Hanes' transformation of the Michaelis-Menten equation: $S/v = K_m/V_{max} + S/V_{max}$. The points represent the mean values of four replicates. The mean values of four equilibrium exchange experiments with 1 S.D. were: $K_m = 5.47 \pm 1.00$ mM; $V_{max} = 2.16 \pm 0.46$ $mM \cdot s^{-1}$. The equivalent values for nine zero-trans experiments were: $K_m = 5.11 \pm 1.87$ mM; $V_{max} = 2.01 \pm 0.64$ $mM \cdot s^{-1}$. The inset shows the time-course of uptake of 10 mM 3-*O*-methylglucose, according to either the equilibrium exchange (●———●) or zero-trans (○———○) protocol. The points represent mean values of three paired experiments.

This suggests symmetrical transport kinetics. Also, there is no trans acceleration, a phenomenon which has been reported in cultured hepatocyte-like cells [12]. The K_m for 3-*O*-methylglucose transport of 5 mM is similar to that obtained in adipocytes at 37°C [13,14] but different from the 20 mM previously obtained in rat hepatocytes at 20°C [4].

Exit experiments were also carried out but the results were less precise because it is necessary first to dilute the cells with a relatively large efflux volume and then stop at very short time intervals. In summary, zero-trans exit K_m was of the same magnitude as that of zero-trans entry; infinite cis exit data were not precise enough to determine whether the K_m was different from the zero-trans exit K_m (data not shown). Other experiments (not demonstrated) showed that neither dexamethasone (1 μM, preincubation time 120 min) nor insulin (1 μM) had any demonstrable effect on 3-*O*-methylglucose entry.

3-*O*-Methylglucose transport was also measured in cells prepared from fed rats and the rate constant of entry of tracer was 0.46 ± 0.05 s^{-1} (1 S.D., $n = 4$). The apparent zero trans entry K_m was 6.1 ± 1.8 mM and the apparent V_{max} was 2.5 ± 1.0 mM $\cdot$ s^{-1}. Glucose was released from the hepatocytes and the concentration in the medium at the time of transport measurement was 2–3 mM. This would not markedly change the measured kinetic constants for 3-*O*-methylglucose (cf. Table I). Therefore, the state of the glucose transport system seems closely similar in hepatocytes from fed and fasted animals.

Transport of metabolizeable aldohexoses

The ability of various sugars to inhibit transport of the ^{14}C-labelled 3-*O*-methylglucose were measured and the inhibition constants (K_i) are shown in Table I together with the rate constants of entry (i.e. V_{max}/K_m) of the labelled sugars. K_i for D-glucose was 19 mM and K_m for D-glucose using ^{14}C-labelled glucose was similarly determined as about 20 mM (data not shown). This is in excellent agreement with the value derived from perfusion experiments by Williams et al. [1]. Both mannose and galactose, at tracer concentrations, showed rate constants of 10–15% of that of 3-*O*-methylglucose and their inhibition constants were accordingly around 50 mM.

Previous studies [3] have shown that the rate of D-glucose transport greatly exceeds the rate of accumulation of labelled products in the cells. This was also true in the present experiments. In fact, accumulation of ^{14}C-labelled products was negligible even after incubation for 10 min (data not shown). However, this rate of product accumulation greatly underestimates the rate of glucose phosphorylation since a large fraction of the glucose 6-phosphate is dephosphorylated in the liver [11]. Therefore, we measured the rate of conversion of [2-^{3}H]glucose to total metabolic products. The rationale for this is that tritium at the two-position is exchanged with water in the hexose phosphate isomerase reaction [11]. Thus, the label is lost from at least the majority of the glucose molecules which are phosphorylated and then dephosphorylated. The rate of phosphorylation of 0.1 mM glucose was measured in this way as $2.5 \cdot 10^{-3}$ s^{-1}, i.e. about 2% of the transport rate. This rate constant remained essentially unchanged when 15 mM glucose was used with a preincubation time of 45 min in the absence or presence of insulin. Other experiments demonstrated that the rate of accumulation of metabolic products from ^{14}C-labelled 2-deoxyglucose or galactose were also much lower than the transport rates of these sugars (data not shown). This is in agreement with previous observations that galactose transport is not rate-determining for galactose metabolism [3].

Fructose transport and metabolism

Perfusion studies in the rat liver have suggested that transport might limit metabolism of fructose at low concentrations [15]. Fig. 3 shows uptake rates at different fructose concentrations ranging from trace (3.4 μM) to 10 mM. Equilibration of 3-*O*-methylglucose is shown for comparison. Uptake of fructose at all concentrations is regarded as largely limited by the transport step at the early times (0–15 s) where the per cent intracellular labelled fructose is considerably lower than the per cent intracellular 3-*O*-methylglucose at equilibrium. It appears that fructose uptake is approximately linear from 0 s to 120 s at the tracer concentration suggesting that transport may be rate-limiting for metabolism at this condition. $^{14}CO_2$ formation from labelled fructose was

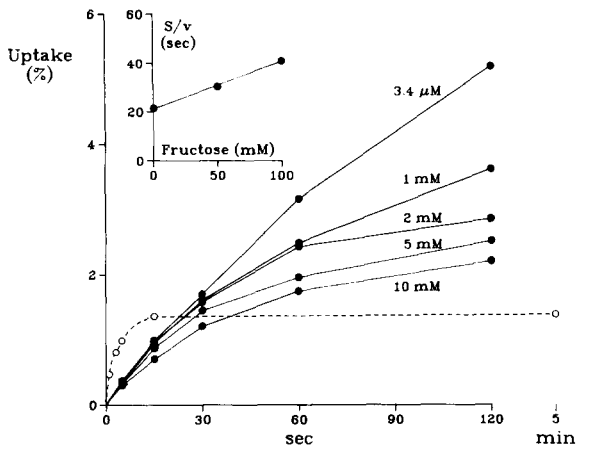

Fig. 3. Time-course of fructose uptake. Each point represents the mean of four replicate values for uptake at various fructose concentrations (●———●). Equilibration of 3-*O*-methylglucose is also shown (○— — —○). The inset shows the concentration dependence of fructose transport measured at times giving fructose uptake corresponding to about 25% of the equilibrium space for 3-*O*-methylglucose. Osmolarity was kept constant by the addition of mannitol. The apparent K_m was 107 mM and V_{max} was 5.1 mM·s^{-1}.

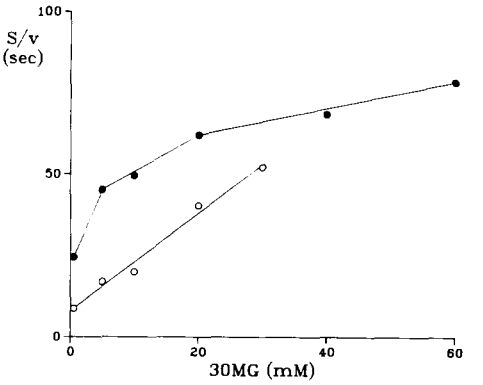

Fig. 4. Inhibition of fructose transport by 3-*O*-methylglucose (30MG). The transport of ^{14}C-labelled fructose (●———●) was measured in the presence of various concentrations of 3-*O*-methylglucose as explained in the legend to Fig. 3, inset. Each point is the mean of four replicate values. For comparison is shown the inhibition of ^{14}C-labelled glucose transport by 3-*O*-methylglucose (○———○) and K_i was measured in four experiments as 5.4 ± 0.5 mM (1 S.D.).

negligible (data not shown). Fructose transport is clearly not rate-limiting with substrate concentrations higher than 1 mM, since the uptake rates were much slower at the late times than at the early times under these conditions. The inset shows approximate K_m and V_{max} values for fructose transport measured at times short enough to calculate initial velocities, cf. legend to Fig. 3.

The experiment shown in Fig. 4 was designed to test whether fructose is transferred exclusively via the same transporter as 3-*O*-methylglucose, i.e. via the glucose transporter. If so, the reciprocal rate constant for labelled fructose transport (S/v) should increase linearly with increasing 3-*O*-methylglucose concentration. As shown in Fig. 4, this relationship is markedly non-linear indicating that fructose is transferred via at least two pathways. Moreover, the rate constant of tracer fructose entry was measured as $8.3 \cdot 10^{-3}$ s^{-1} in the presence of a saturating concentration of 3-*O*-methylglucose (150 mM = 30-times K_m) as compared with a value of about $1.2 \cdot 10^{-3}$ s^{-1} calculated under the assumption that fructose was only taken up via the glucose transporter. The inhibition of transport of labelled glucose by 3-*O*-methylglucose is shown for comparison. The rate constant of tracer fructose entry in the absence of 3-*O*-methylglucose was calculated from the experiment shown in Figs. 4 and 5 similar experiments as $4.3 \cdot 10^{-2} \pm 0.4 \cdot 10^{-2}$ s^{-1} (1 S.D.). This is about 100-times higher than the rate constant for L-glucose entry, cf. Table I. It is therefore very unlikely that fructose is transferred by nonmediated diffusion to any significant extent. The experiments therefore strongly suggest that fructose is transferred both via the glucose transporter, albeit with low affinity, and via a low affinity fructose transporter.

Discussion

The ability to measure the initial velocity of a nonmetabolizeable glucose analogue such as 3-*O*-methylglucose is a prerequisite for the evaluation of kinetic parameters. 3-*O*-methylglucose at a concentration much lower than K_m equilibrates with a half time of less than 2 s in the hepatocytes at physiological temperature (Fig. 1). Previous studies at 37°C [3,16] have been carried out using methods which do not allow sufficiently rapid sampling.

Craik and Elliot [4] evaluated the hexose transport system using 3-*O*-methylglucose at 20°C and

found a half-time of equilibration of about 8 s at low substrate concentration. K_m for 3-*O*-methylglucose equilibrium exchange was determined as about 20 mM. The present V_{max} for 3-*O*-methylglucose of 2.2 $mM \cdot s^{-1}$ is approx. 70% higher than the value determined at 20°C [4]. The present K_m of about 5 mM is not distinguishable from that reported in rat [13,14] and human [17] adipocytes, and in human polymorphonuclear leukocytes [10] at 37°C. The decrease in K_m at higher temperature seems also to be reflected in the results on galactose, cf. the present K_i of 53 mM (Table I) versus a K_m of 174 mM at 20°C [5]. The K_m for glucose of 20 mM is in excellent agreement with the early perfusion study by Williams et al. [1].

The kinetic parameters of both equilibrium exchange, zero-trans entry and zero-trans exit were assessed in the previous study at 20°C and the transport system was found to be symmetrical [4]. This conclusion is supported by the close similarity between zero-trans entry and equilibrium exchange entry at 37°C (Fig. 2). In fact, the major differences are seen between the results of these protocols in cells with markedly asymmetric transport systems such as human erythrocytes (at least at 25°C) and human cultured lymphocytes [9].

The present results with fructose show that transport exceeds metabolism when the substrate concentration is 1 mM or higher. In contrast, previous experiments at 20°C showed a metabolism rate of 8.8 mM fructose similar to the transport rate [5]. Furthermore, we have found that fructose is taken up not only via the glucose transporter but also via another facilitated transport system. This system seems of minor importance when the extracellular 3-*O*-methylglucose (or glucose) concentration is low since the transport of labelled fructose was reduced to 15–20% in the presence of a saturating 3-*O*-methylglucose concentration. However, it will allow the hepatocyte to take up fructose even in the presence of very high glucose concentrations. The nature of this system is unknown but it may be similar to the fructose transporter described in rat adipocytes [18]. The hepatocyte seems similar to the insulin stimulated adipocyte which transfers most of the fructose via the abundant glucose transporters in spite of the simultaneous presence of fructose transporters [18].

In summary, K_m for 3-*O*-methylglucose is about 5 mM. K_m for glucose is about 20 mM and the rate of transport is about 50-times higher than the rate of phosphorylation. Fructose is taken up both via the glucose transporter and via a tentative fructose transporter, and fructose transport exceeds its metabolism when the concentration is higher than 1 mM.

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